

# Regionalized Transcriptional Domains of Myosin Light Chain 3f Transgenes in the Embryonic Mouse Heart: Morphogenetic Implications

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Within the embryonic heart, five segments can be distinguished: two fast-conducting atrial and ventricular compartments flanked by slow-conducting segments, the inflow tract, the atrioventricular canal, and the outflow tract. These compartments assume morphological identity as a result of looping of the linear heart tube. Subsequently, the formation of interatrial, interventricular, and outflow tract septa generates a four-chambered heart. The lack of markers that distinguish right and left compartments within the heart has prevented a precise understanding of these processes. Transgenic mice carrying an *nlacZ* reporter gene under transcriptional control of regulatory sequences from the MLC1F/3F gene provide specific markers to investigate such regionalization. Our results show that transgene expression is restricted to distinct regions of the myocardium:  $\beta$ -galactosidase activity in 3F-*nlacZ*-2E mice is confined predominantly to the embryonic right atrium, atrioventricular canal, and left ventricle, whereas, in 3F-*nlacZ*-9 mice, the transgene is expressed in both atrial and ventricular segments (right/left) and in the atrioventricular canal, but not in the inflow and outflow tracts. These lines of mice illustrate that distinct embryonic cardiac regions have different transcriptional specificities and provide early markers of myocardial subdivisions. Regional differences in transgene expression are not detected in the linear heart tube but become apparent as the heart begins to loop. Subsequent regionalization of transgene expression provides new insights into later morphogenetic events, including the development of the atrioventricular canal and the fate of the outflow tract.

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## INTRODUCTION

In mammalian embryos the precardiac mesoderm, or cardiogenic plate, occupies a horseshoe-shaped area at the rostral end of the germ disc. The two dorsolateral edges of the cardiogenic plate migrate toward the midline of the body axis and fuse, in a craniocaudal direction, into a single tube (see Manasek, 1968; Van Mierop, 1979). As soon as the precardiac mesoderm is formed, the cells begin to express muscle-specific proteins including SERCA2, myosin heavy chain (MHC), myosin light chain (MLC), actin, tropomyosin, and various intermediate filament isoforms (Schaart *et*

*al.*, 1989; Van der Loop *et al.*, 1992; Ruzicka and Schwartz, 1988; Barton *et al.*, 1988; Lyons *et al.*, 1990; Moorman *et al.*, 1995). In the chicken heart tube an atrial-specific marker is already activated specifically in the posterior part of the tube, although other chamber-specific markers do not show this early compartmentalization (Yutzey *et al.*, 1994; Yutzey and Bader, 1995); indeed, *in vivo* labeling techniques in the chicken suggest that the cardiac tube contains only ventricular primordia (De la Cruz *et al.*, 1989). In mammals, it is not clear whether the expression of chamber-specific myocardial genes shows spatial restriction at this stage.

With further development, the primitive cardiac tube bends to the right-hand side and newly formed myocardium is added at both ends of the tube (De la Cruz *et al.*, 1989). At this stage the atrial and ventricular compartments can first be distinguished. The cardiac tube is formed of two layers, endocardium and myocardium, separated by extracellular matrix, the cardiac jelly (Davis, 1927). The myocardium is characterized by gradients of gene expression along

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its length.  $\alpha$ -MHC is highly expressed in the inflow tract region and decreases toward the outflow tract (Wessels *et al.*, 1991). In contrast,  $\beta$ -MHC shows the opposite pattern of expression, since it is highly expressed in the outflow and decreases toward the inflow tract (De Groot *et al.*, 1989; Wessels *et al.*, 1991).

During further development, the cardiac jelly becomes confined to the outflow tract and atrioventricular canal (Manasek, 1976), and the ventricle acquires a trabeculated morphology (Ben-Sahchar *et al.*, 1985). During this process, changes in the pattern of gene expression occur which are correlated with differences in function (De Jong *et al.*, 1992; Moorman and Lamers, 1994). Five segments can be distinguished; two fast-conducting atrial and ventricular compartments flanked by slow-conducting segments, the inflow tract, the atrioventricular canal, and the outflow tract. The atrial and ventricular compartments are characterized by the expression of specific MHC isoforms,  $\alpha$  and  $\beta$ , respectively (De Groot *et al.*, 1989; Wessels *et al.*, 1991). The flanking segments coexpress both isoforms (De Groot *et al.*, 1989). The expression of regulatory and alkali MLC isoforms also becomes confined to distinct compartments (see Lyons, 1994). MLC1A and MLC2A mRNAs are mostly restricted to the atrial myocardium (Lyons *et al.*, 1990; Kubalak *et al.*, 1994) and MLC1V and MLC2V mRNAs to the ventricular compartment (Lyons *et al.*, 1990; O'Brien *et al.*, 1993). MLC3F mRNA is also present in embryonic cardiac muscle, being more abundant in the atria than the ventricles (Kelly *et al.*, 1995). Changes in the spatial expression of myocardial genes occur asynchronously during development (Lyons *et al.*, 1990).

Correct development of the heart involves the formation of three different septa that converge in the lesser curvature of the heart: the aorticopulmonary septum, the interatrial septum, and the interventricular septum. The process of septation is a crucial event during cardiogenesis and many abnormalities originate from a failure of this process (Becker and Anderson, 1983). The outflow tract is initially connected with the embryonic right ventricle and the common atrium is connected with the embryonic left ventricle. With further development, the left ventricular chamber obtains an independent arterial connection. Whereas the specification of atrial and ventricular compartments has been extensively studied (Yutzey and Bader, 1995), little is known about the origins of right and left ventricular specification and how the new connections are made, i.e., the left arterial and right atrioventricular connections. The lack of markers that distinguish right and left cardiac compartments has prevented a precise understanding of the septation process. The generation of transgenic mice carrying an *nlacZ* reporter gene under the transcriptional control of regulatory sequences of the MLC1F/3F gene provides a tool to unravel these processes.

We describe here a detailed analysis of the pattern of *nlacZ* expression during cardiac embryogenesis in two lines of MLC3F transgenic mice. 3F-*nlacZ*-2E mice contain 2 kilobases (kb) of DNA sequence upstream of the MLC3F transcription initiation site together with a 3' skeletal muscle-

specific enhancer element (Donoghue *et al.*, 1988; Rosenthal *et al.*, 1989; Kelly *et al.*, 1995). 3F-*nlacZ*-9 mice contain 9 kb of sequence upstream from the MLC3F transcriptional initiation site and no 3' enhancer. The 9-kb sequence includes a second muscle-specific enhancer, present in the first intron of the gene (Kelly *et al.*, 1997). Transgene expression in 3F-*nlacZ*-2E mice reveals a distinction between left and right sides of the heart. Expression is mainly confined to the embryonic right atrium, atrioventricular canal, and left ventricle, whereas in 3F-*nlacZ*-9 mice expression is restricted to both atrial and ventricular cavities (right/left) and the atrioventricular canal, but is not observed in the outflow or inflow tract regions. These lines of mice illustrate that distinct embryonic cardiac segments acquire different transcriptional specificities as the heart loops and provide early markers of subdivisions of the myocardium with implications for cardiac morphogenesis.

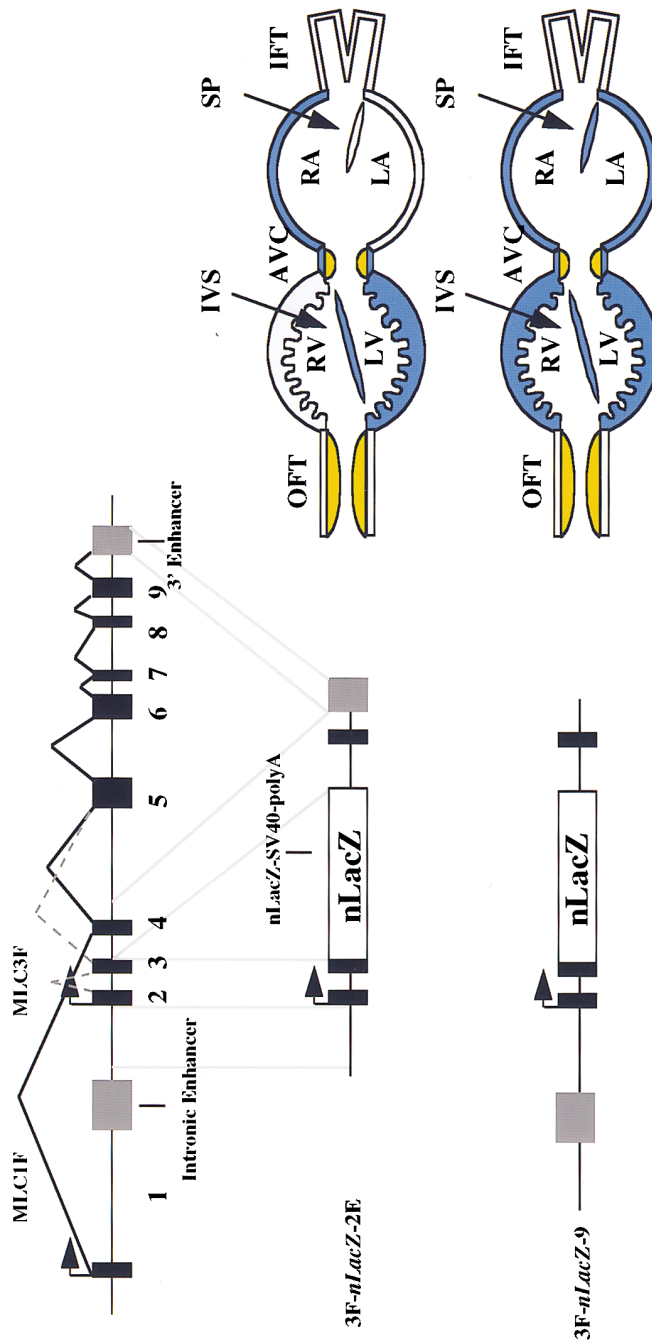
## MATERIALS AND METHODS

### Transgenic Mice

Two MLC3F transgenic lines containing the *nlacZ* reporter gene under the transcriptional control of regulatory elements of the MLC3F gene were analyzed. Construct 3F-*nlacZ*-2E (Kelly *et al.*, 1995) contains 2 kb of upstream sequence in front of the MLC3F promoter driving expression of an *nlacZ* reporter gene and the 3' MLC1F/3F enhancer element placed 3' to the polyadenylation site (Fig. 1). Construct 3F-*nlacZ*-9 (Kelly *et al.*, 1997) contains a 9-kb fragment of DNA upstream of the MLC3F transcription initiation site driving the expression of the *nlacZ* reporter gene (Fig. 1). Details of transgene construction and the characterization of skeletal muscle expression during development and in the adult in these transgenic lines are reported elsewhere (Kelly *et al.*, 1995, 1997). In this study data are presented for one line of each transgene construct (3F-*nLacZ*-2E, line 1, and 3F-*nLacZ*-9, line 9a). In both cases a second transgenic line gave similar results.

### Embryos

Heterozygous and homozygous adult specimens and embryos ranging from Embryonic Day (E) 7 to E16 for each transgenic line (crossed with nontransgenic C57BL6/J  $\times$  SJL F1 females) were analyzed. The day of plug was taken as E0.5. Embryos were excised from the uterus and the thoracic wall was removed (E12.5 to E16.5), exposing the heart to allow maximal penetration of fixatives and reagents. Adult hearts were dissected at the arterial and venous pole of the heart, keeping intact the caval and pulmonary veins. Specimens were briefly fixed in freshly prepared 4% paraformaldehyde for *in situ* hybridization (4 hr) or in methanol:acetone:water for immunohistochemistry (30 min–4 hr) and rinsed twice in phosphate-buffered saline (PBS). For *in toto*  $\beta$ -galactosidase histochemistry, or *in toto*  $\beta$ -galactosidase histochemistry followed by immunohistochemistry, specimens were directly processed as detailed below. Specimens were rinsed in increasing sucrose gradients (10, 20, and 30% in PBS) for 2 hr at each step, embedded in OTC (Miles Inc., USA), and frozen. Freeze cryotome serial sections of 7–10  $\mu$ m were cut, mounted onto gelatin-coated slides, and stored at  $-20^{\circ}\text{C}$  until use. Embryos for *in situ* hybridization were dehydrated in increasing concentrations of ethanol and embedded in paraffin. Se-



**FIG. 1.** MLC3F transgene constructs and the pattern of cardiac expression at E10. Schematic representation of the MLC1F/3F locus showing the transcriptional start sites (—), exon (■) structure, MLC1F (—), and MLC3F (---) splicing patterns and the position of two skeletal muscle enhancer elements (grey bars). MLC3F transgene constructs are shown with transgene expression patterns at approximately Embryonic Day (E) 10. Blue represents transgene expression and yellow the endocardial cushions of the outflow tract and the atrioventricular canal. OFT, outflow tract; AVC, atrioventricular canal; IFT, inflow tract; RA, right atrium; LA, left atrium; RV, right ventricle; LV, left ventricle; IVS, interventricular septum; SP, septum primum.

**TABLE 1**  
Transgene Expression in the Developing Mouse Heart in 3F-*nLacZ*-2E and 3F-*nLacZ*-9

Structure	Lines:	Embryonic		Fetal		Adult	
		2E	9	2E	9	2E	9
Outflow tract		—	—	—	—		
Right ventricle		—	+	—	+	—	+
Interventricular septum		+	+	+	+	+	+
Left ventricle		+	+	+	+	+	+
Atrioventricular canal		+	+				
Right atrium		+	+	+	+	+	+
Left atrium		—	+	—	+	—	+
Septum primum				—	—		
Septum secundum				—	—		
Interatrial septum						—	—
Sinus horns		+	+				
Coronary sinus				—	—	—	—
Caval veins				—	—	—	—
Pulmonary veins				—	—	—	—

*Note.*  $\beta$ -Galactosidase expression in the embryonic, fetal, and adult cardiac structures in 3F-*nLacZ*-2E (2E) and 3F-*nLacZ*-9 (9) transgenic mice. (—) no expression, (+) positive expression. Where no indication is given, the structure does not exist at the stage in question.

rial sections of 7  $\mu$ m were cut, mounted onto RNase-free aminopropyltriethoxysilane-coated slides, and stored at room temperature.

***In Toto X-Gal Histochemical Staining***

Specimens were briefly fixed in freshly prepared 4% paraformaldehyde (30 min – 1 hr) before histochemical detection of  $\beta$ -galactosidase. Incubation in X-gal solution at 37°C was performed for periods of 30 min to overnight as detailed elsewhere (Sanes *et al.*, 1986). Subsequently, whole-mount embryos and adult hearts were post-fixed in freshly made 4% paraformaldehyde for 4 hr to overnight and conserved in 70% ethanol until analyzed.

***In Toto  $\beta$ -Galactosidase Staining and Immunohistochemistry***

The specimens were first stained with X-gal as described above, rinsed twice in PBT (PBS containing 0.1% Tween 20) for 5 min, and then processed for immunohistochemistry. The embryos were treated for 4–5 hr with hydrogen peroxide (3% in PBT) to reduce endogenous peroxidase activity. They were then incubated with PBTMT (1% defatted milk powder in PBT) for 2 hr to avoid nonspe-

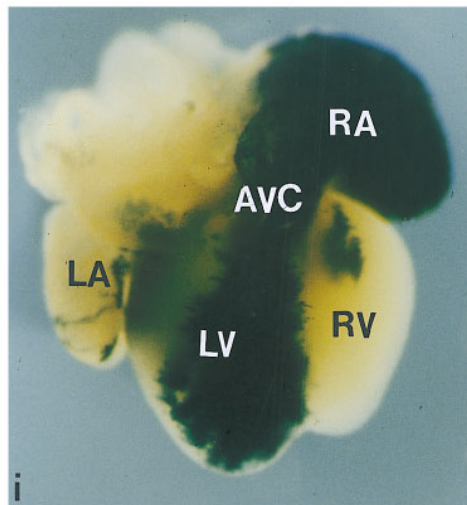
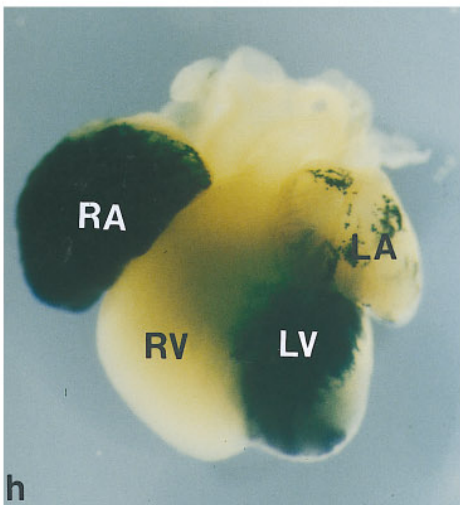
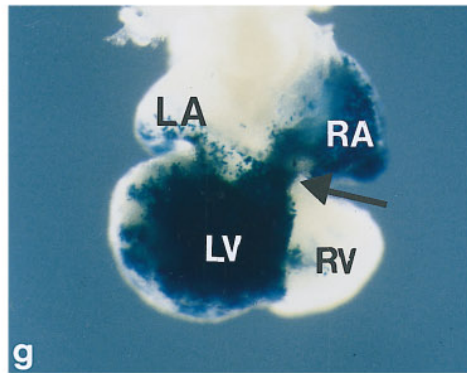
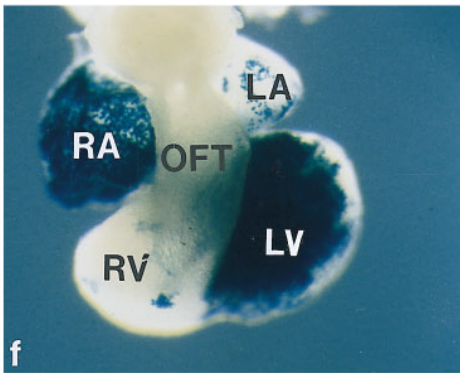
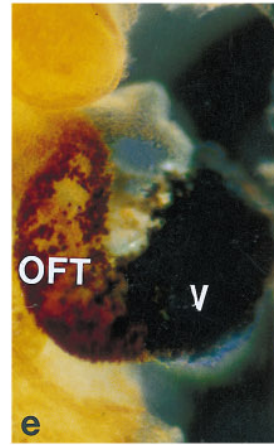
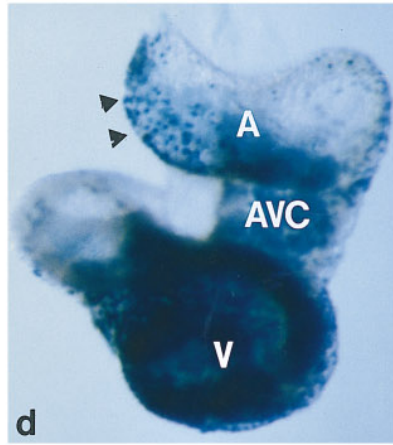
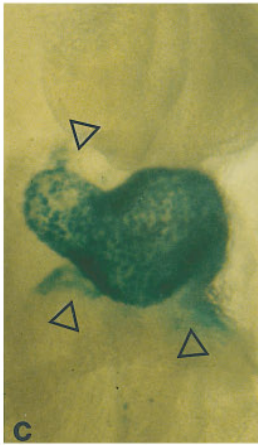
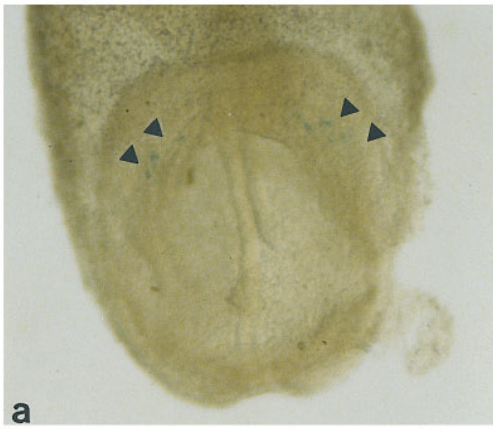
cific binding of the primary antibody. After this pretreatment, whole-mount embryos were washed again in PBTMT and incubated overnight with primary antibody diluted in PBTMT (1:20). A specific primary polyclonal antibody recognizing all myosin heavy chain isoforms was used (Cusella de Angelis *et al.*, 1994) to delineate the myocardium. First antibody binding was detected by the uncoupled peroxidase–antiperoxidase method as described elsewhere (Dent *et al.*, 1987).

***$\beta$ -Galactosidase Detection and Immunohistochemistry in Cryosections***

Sections were rinsed briefly in PBS and incubated at 37°C (1 hr – overnight) in X-gal reagent. After a brief rinse in PBS for 5 min, alternative sections were washed in bidistilled water for 5 min, counterstained with azofloxine or eosin (1%) for 5 min, dehydrated, and mounted in Entellan (Merck); other sections were processed for immunohistochemical detection of cardiac-specific antigens.

Specific primary monoclonal antibodies against human  $\alpha$ -MHC and  $\beta$ -MHC (Wessels *et al.*, 1991) were used to visualize atrial and ventricular myocardium, respectively. After treatment with hydrogen peroxide (3% in PBS, 30 min) to reduce endogenous perox-

**FIG. 2.** Developmental profile of transgene expression in the 3F-*nLacZ*-2E mice. *In toto* localization of  $\beta$ -galactosidase in 3F-*nLacZ*-3E transgenic embryos. (a) Labeling is distributed symmetrically (arrowheads) in the precardiac mesoderm (E7.5) and (b) early tubular heart stages (E8). (c) In the looping heart (E8.5),  $\beta$ -galactosidase activity is observed throughout the cardiac tube; however, it is reduced at the poles (arrowheads). (d) As development proceeds (E9.5), expression in the atrium (A) becomes preferentially restricted toward the right side (arrowheads). (e) Whole-mount immunocytochemistry using an anti-MHC antibody on an E9.5 embryo demonstrates that whereas the common ventricle (V) is strongly  $\beta$ -galactosidase positive, few  $\beta$ -galactosidase cells are present in the outflow tract (OFT) which is positive for the myocardial marker. (f) and (g) correspond to an E10.5 embryo in ventral and dorsal views, respectively. Transgene expression is confined to the left ventricle (LV) and right atrium (RA) (f), forming a continuum of  $\beta$ -galactosidase cells (arrow) along the atrioventricular canal (AVC) (g). (h) and (i) correspond to an E14.5 heart in dorsal and ventral views, respectively. The pattern of expression is essentially identical to E10.5. LA, left atrium; RV, right ventricle.



idase activity, an incubation in TENG-T (10 mM Tris, 5 mM EDTA, 150 mM NaCl, 0.25% gelatin, 0.05% Tween 20, pH 8.0; 30 min) was performed to avoid nonspecific binding. The sections were then incubated overnight with specific primary antibodies. Binding of the first antibody was detected using a rabbit anti-mouse immunoglobulin, followed by a goat anti-rabbit immunoglobulin and finally a rabbit peroxidase anti-peroxidase (PAP) complex. Each incubation lasted 2 hr and was followed by three washes in PBS (5 min each). All sera were diluted in PBS. The visualization of the PAP complex was performed by incubation with 0.5 mg/ml 3,3'-diaminobenzidine and 0.02% hydrogen peroxide in 30 mM imidazole, 1 mM EDTA (pH 7.0) buffer. Sections were dehydrated and mounted in Entellan (Merck).

## In Situ Hybridization

Complementary RNA probes against rat  $\alpha$ -MHC (Schiaffino et al., 1989; Boheler et al., 1992), rat  $\beta$ -MHC (Boheler et al., 1992), MLC2A (Kubalak et al., 1994), MLC2V (O'Brien et al., 1993), and  $\beta$ -galactosidase (Kelly et al., 1995) mRNAs were radiolabeled with [<sup>35</sup>S]UTP by *in vitro* transcription according to standard protocols (Melton et al., 1984). Hybridization conditions were as detailed elsewhere (Moorman et al., 1993, 1995). Briefly, the sections were deparaffined, rinsed in absolute ethanol, and dried in an air stream. Pretreatment of the sections was as follows: 20 min 0.2 N HCl, 5 min bidistilled water, 20 min 2× SSC (70°C), 5 min bidistilled water, 2–20 min digestion in 0.1% pepsin dissolved in 0.01 N HCl, 30 sec in 0.2% glycine/PBS, two 30-sec rinses in PBS, 20 min of postfixation in freshly prepared 4% paraformaldehyde, 5 min in bidistilled water, 5 min in 10 mM EDTA, 5 min in 10 mM DTT, and finally drying in an air stream. The prehybridization mixture contained 50% formamide, 10% dextran sulfate, 2× SSC, 2× Denhardt's solution, 0.1% Triton X-100, 10 mM DTT, and 200 ng/ $\mu$ l heat-denatured herring sperm DNA. The sections were hybridized overnight at 52°C and washed as follows: a rinse in 1× SSC, 30 min at 52°C in 50% formamide dissolved in 1× SSC, 10 min in 1× SSC, 30 min in RNase A (10  $\mu$ g/ml), 10 min in 1× SSC, 10 min in 0.1× SSC, and dehydration in 50, 70, and 90% ethanol containing 0.3 M ammonium acetate. The sections were then dried and immersed in nuclear autoradiographic emulsion G5 (Ilford). The exposure times ranged from 7 to 14 days and the development times from 4 to 8 min. Photographs were taken with a Zeiss Axiophot microscope, using Agfa 25ASA films.

## RESULTS

3F-*nlacZ*-2E and 3F-*nlacZ*-9 transgenic mice express a  $\beta$ -galactosidase reporter gene with a nuclear localization signal (*nlacZ*) in skeletal and cardiac striated muscle. Nuclear localization improves the resolution of detection at the single-cell level. This study is restricted to the analysis of transgene expression in the myocardium, in both embryonic and adult transgenic mice. Cardiac development is considered over three periods: the first covers the period from the precardiac mesoderm stage up to the looping heart (early embryonic period), the second comprises the development of right/left components by the process of atrioventricular septation (embryonic period), and the third includes septation of the heart and acquisition of the mature cardiac architecture (fetal and adult period). Our results are summarized

in Fig. 1 and Table 1. The 3F-*nlacZ*-2E line shows striking predominance of expression in the left ventricle and right atrium, whereas in the 3F-*nlacZ*-9 line both ventricles and atria express the transgene, but the outflow and inflow tracts are negative. Specific attention is given to transgene expression during the processes of atrioventricular canal septation (3F-*nlacZ*-2E line) and outflow tract septation (3F-*nlacZ*-9 line). The data presented show the expression pattern of the transgene in heterozygous mice; homozygous animals displayed similar patterns of expression (data not shown).

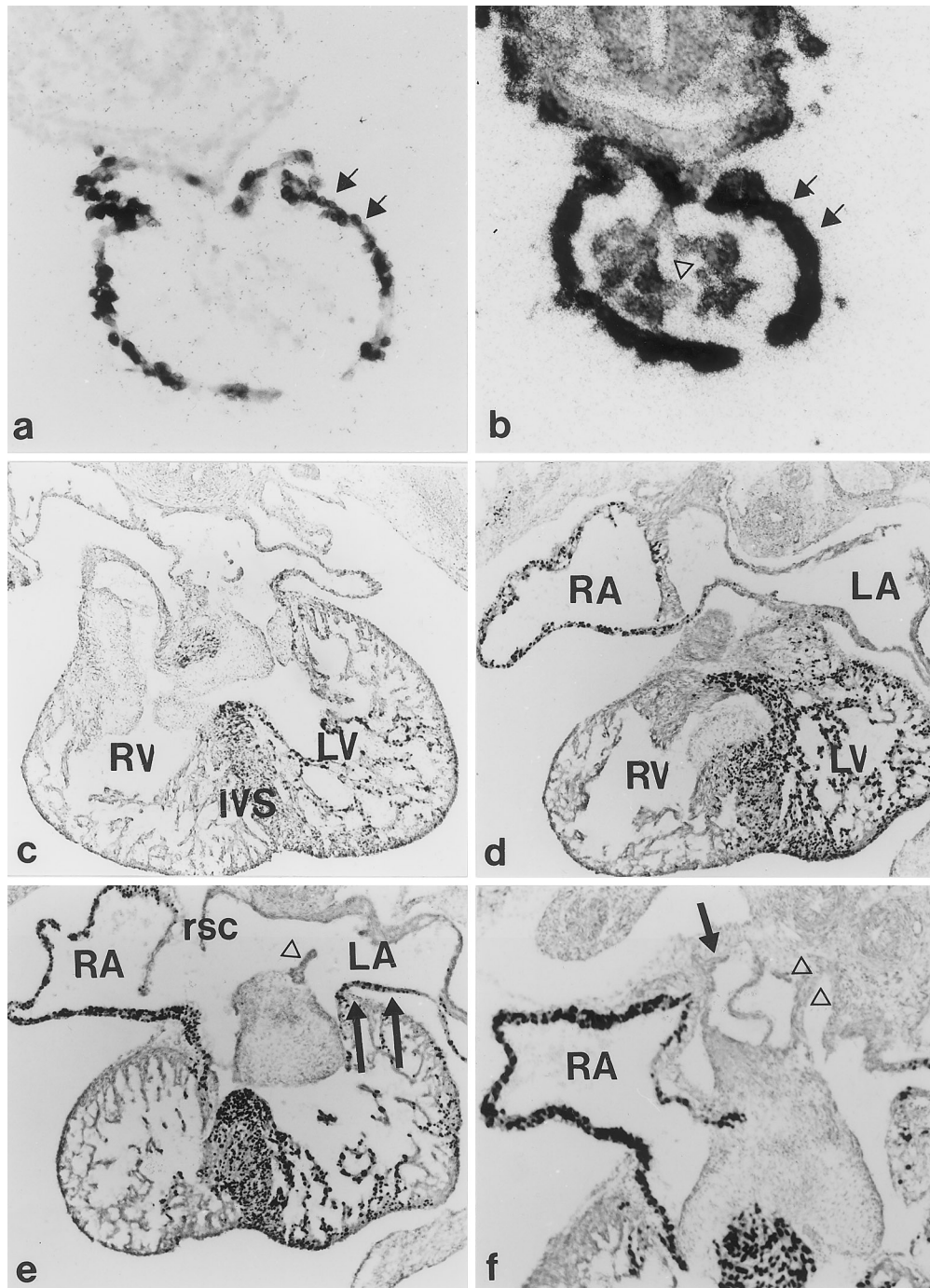
## 3F-*nlacZ*-2E Transgenic Mice

3F-*nlacZ*-2E transgenic mice contain *nlacZ* under transcriptional control of the MLC3F promoter (2 kb of sequence upstream from the transcriptional start site) and a 3' enhancer element (Kelly et al., 1995). Two 3F-*nlacZ*-2E transgenic lines show indistinguishable expression patterns. The overall adult cardiac expression in these mice has been reported previously and shows that transcriptional specificities differ in the right and left ventricular chambers (Fig. 1; Kelly et al., 1995). In this study we follow the development of these differences during embryogenesis.

**Expression in the precardiac mesoderm and the primitive cardiac tube (E7.5–E9.5).** At the cardiogenic plate stage (E7.5), a few cells expressing the *nlacZ* reporter gene are observed symmetrically located on both sides of the anteroposterior axis (Fig. 2a). These areas fuse in an anteroposterior direction at the midline of the body to form a single cardiac tube in which  $\beta$ -galactosidase-positive cells are symmetrically distributed (E8, Fig. 2b). *nlacZ* expression extends from the embryonic atrium through the atrioventricular junction and common ventricle (Fig. 2c). The extremities of the heart tube are initially  $\beta$ -galactosidase positive, expression colocalizing with that of  $\beta$ -MHC and SERCA2 mRNAs at the venous and arterial myocardial boundaries, respectively (as assessed by *in situ* hybridization to serial transverse sections, Figs. 3a and 3b). The first signs of regionalization are observed at E8.5 as the heart starts to loop to the right-hand side. Negative myocytes are observed at the distal arterial pole adjacent to the aortic sac and at the venous pole at the entrance of the sinus horns (Fig. 2c); *nlacZ*-expressing cells are located in the proximal part of the right and left sinus horns, facing the common atrium (Figs. 2c, 3a, and 3b). The  $\beta$ -galactosidase-negative areas at the arterial and venous poles probably represent the earliest primordia of the outflow tract and inflow tract, respectively.

At E9–9.5 five functional segments can be distinguished: outflow tract, common ventricle, atrioventricular canal, common atrium, and inflow tract (Moorman and Lamers, 1994). Expression of  $\beta$ -galactosidase is observed in the ventricle, atrioventricular canal, and common atrium (Fig. 2d), but not in the outflow tract or inflow tract, which are negative for transgene expression although positive on incubation with an anti-myosin antibody (Fig. 2e). Although the common ventricular cavity is  $\beta$ -galactosidase positive,





**FIG. 3.** Histological analysis of transgene expression in 3F-*nlacZ*-2E mice. Histochemical localization of  $\beta$ -galactosidase activity in cryostat sections of hearts corresponding to E8.5 (a, b), E11.5 (c, d), and E12.5 (e, f) embryos. (b) Myocardial cells at the venous pole of the heart in an adjacent serial section to (a), as revealed by *in situ* hybridization to SERCA2 mRNA. At E8.5 all  $\beta$ -galactosidase-positive nuclei (arrows) are myocardial (a), whereas endocardial cells are not labeled (arrowhead) (b). In sections through all four cardiac compartments at E11.5,  $\beta$ -galactosidase activity is restricted predominantly to the left ventricle (LV), interventricular septum (IVS), and right atrium (RA) (c, d). At E12.5,  $\beta$ -galactosidase activity is confined to the LV, IVS, and RA up to the entrance of the right superior caval vein (rsc). In the left atrium (LA), the myocardium in close apposition to the atrioventricular junction is also positive for the transgene (arrows) (e). In contrast, structures such as the septum primum (arrowhead) (e), caval veins (arrow) (f), and coronary sinus (arrowheads) (f) do not show  $\beta$ -galactosidase activity.

more positive cells are observed in the left side than the right side of the primitive ventricular myocardium (data not shown). In the atrium at this stage transgene expression is higher in the right side than the left (Fig. 2d). Thus, prior to septation, signs of right–left differentiation are already detectable in the embryonic heart.

**Expression pattern in the early septating heart (E10.5–E12.5).** From E10.5 onward the common ventricle and atrium become separated into right and left components, preceding septation of the outflow tract. The outflow tract (Fig. 2f) and embryonic right ventricle show almost no staining, whereas the left ventricle is positive (Figs. 2f, 2g, and 3d), including the interventricular septum (Figs. 3c and 3d). At the venous pole of the heart, the dorsal wall of the atrium shows an outgrowth, the septum primum, which first delimits the right and left atrial components (Kauffman, 1992). Transgene expression is confined to the atrioventricular canal (Fig. 2g) and the right atrium (Fig. 3d), whereas the left atrium (Fig. 2g) and the septum primum are negative. In a dorsal view it can be seen that  $\beta$ -galactosidase-positive cells are found along the inner curvature of the heart (atrioventricular canal) extending from the left ventricle to the right expansion of the common atrium (Fig. 2g).

At E12.5, the formation of the interventricular septum is more advanced. The outflow tract is entirely negative and only a few positive cells are located in the embryonic right ventricle; the embryonic left ventricle and the interventricular septum express the transgene (Fig. 3e). At this stage of development the septum primum is clearly identifiable in the atrial cavity and shows no expression of the transgene (Fig. 3e).  $\beta$ -galactosidase-positive cells are observed in the embryonic right atrium up to the leaflets of the sinus venosus; the caval veins and coronary sinus are  $\beta$ -galactosidase negative (Figs. 3e and 3f). In the left atrium very few cells express the transgene, notably at the lower myocardial rim directly opposed to the atrioventricular endocardial cushions. Hence, expression in the embryonic left ventricular component and right atrial side of the embryonic atrium forms a continuum along the inner curvature of the heart. The 3F-*nlacZ*-2E transgene expression pattern remains, in essence, similar from this stage onward.

**Expression in the fetal (E13–E16) and adult heart.** During fetal stages,  $\beta$ -galactosidase-positive cells in 3F-*nlacZ*-2E transgenic mice are restricted mostly to the left ventricle and right atrium (Figs. 2h and 2i). The outflow tract remains negative for transgene expression during septation into right and left components (E13–E16). At the late fetal period a

thin rim of myocardial cells around the left semilunar valve is  $\beta$ -galactosidase negative (data not shown). The right ventricular compartment contains a few positive cells adjacent to the apex of the heart (Fig. 2h), whereas the interventricular septum and the left ventricle are mostly positive. The precise labeling pattern shows some individual variation within the transgenic lines. At the venous pole of the heart, the myocardium surrounding the caval veins, pulmonary veins, coronary sinus, and the dorsal myocardial wall of the atrium, including the septum primum and the developing septum secundum, are  $\beta$ -galactosidase negative (data not shown; see also Fig. 3f).

In the adult heart, the left ventricle is mostly positive, whereas transgene expression is excluded from the right ventricle (Kelly et al., 1995). The entire right atrium expresses the transgene at a high level, except for the myocardium of the caval veins and the coronary sinus, whereas the left atrium, including the pulmonary myocardium, is negative, apart from a few weakly  $\beta$ -galactosidase-positive cells (data not shown).

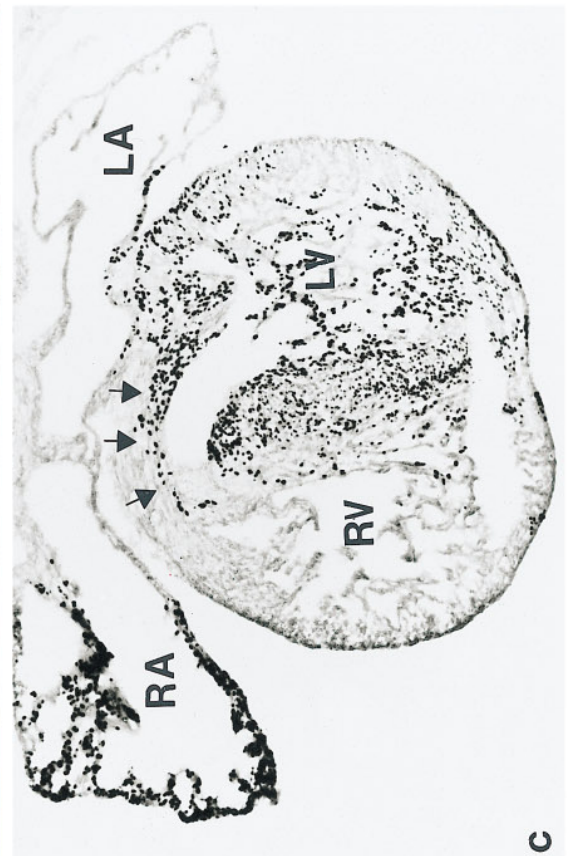
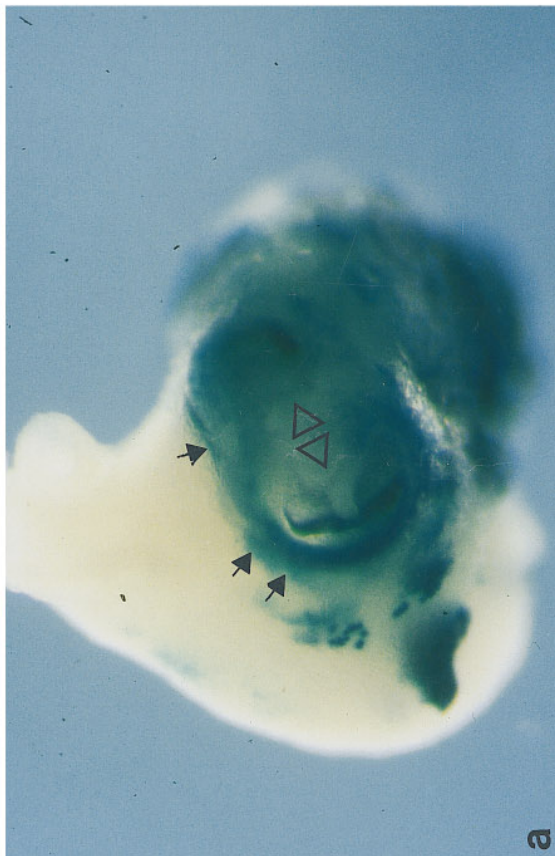
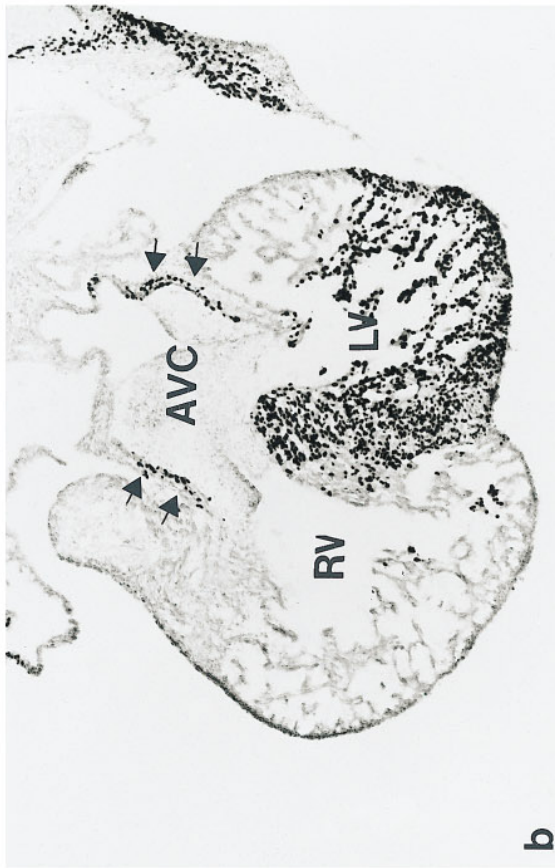
**Transgene expression in the developing atrioventricular canal.** The morphology of the atrioventricular canal changes during alignment with left and right ventricular compartments and atrioventricular valve formation. At E12.5, the atrioventricular endocardial cushions are not yet fused but are in close apposition (Kauffmann, 1992). At this stage cells in the atrioventricular canal myocardium are  $\beta$ -galactosidase positive and form a continuum between right atrium and left ventricle (Figs. 4a and 4b). With further development (E14.5), some  $\beta$ -galactosidase-positive cells are observed in the right ventricle which probably constitute part of the atrioventricular canal. This positive area is initially continuous with positive cells of the left ventricle (Fig. 4c). Caudally, positive cells are observed at the right atrioventricular junction underneath the right atrioventricular endocardial cushions (Fig. 4d). It is important to note that part of the left atrial myocardium adjacent to the forming atrioventricular valve is also  $\beta$ -galactosidase positive (see Fig. 3e). Continuity of transgene expression between the left ventricle and right atrium is no longer observed at this stage due to insulation of the atrial and ventricular myocardium by connective tissue (data not shown).

### 3F-*nlacZ*-9 Transgenic Mice

**Expression in the cardiogenic plate and the primitive cardiac tube (E7.5–E9.5).** 3F-*nlacZ*-9 transgenic mice con-

**FIG. 4.** Development of the atrioventricular canal in 3F-*nlacZ*-2E transgenic mice. (a) *In toto* visualization of transgene expression in the atrioventricular canal (AVC) at E12.5 in 3F-*nlacZ*-2E mice. The atria have been removed by dissection, thus allowing direct observation of the endocardial cushions (arrowheads). Myocardial cells of the AVC are  $\beta$ -galactosidase positive (arrows). Histochemical detection of  $\beta$ -galactosidase activity in transverse sections of E12.5 (b) and E14.5 (c, d) hearts of the 3F-*nlacZ*-2E mice at the level of the lesser curvature of the heart. In addition to positive cells in the right atrium (RA) and left ventricle (LV) the myocardium surrounding the atrioventricular endocardial cushions is positive for  $\beta$ -galactosidase activity (arrows) (b). At E14.5 some positive cells bridge the lesser curvature of the heart (arrows) (c) and more dorsally are found underneath the right atrioventricular cushion tissue (arrows) (d). RV; right ventricle; LA, left atrium.





tain 9 kb upstream of the MLC3F transcriptional start site fused to *nlacZ* in the absence of the 3' enhancer element. Early 3F-*nlacZ*-9 transgene expression, from the cardiogenic plate stage up to the formation of a primitive cardiac tube, is similar to that observed in 3F-*nlacZ*-2E mice (compare Figs. 5a and 2c). Once the cardiac tube is formed and looping initiated (E8.5), cardiomyocytes expressing  $\beta$ -galactosidase are concentrated in the common ventricular region of the tube (Fig. 5a). As the heart continues to loop (E9–E10), the numbers of  $\beta$ -galactosidase-positive cells are reduced at the arterial pole (Fig. 5b). The first clearly distinguishable differences between these transgenes in the developing heart are observed after E9.

**Transgene expression in the looped heart delimits the boundary between the embryonic right ventricle and the outflow tract (E10.5–E12.5).** At E10.5 cells expressing the transgene are observed in the atrial myocardium, the atrio-ventricular canal, and the right and left trabeculated components of the embryonic ventricle. The outflow tract of the heart is negative (Fig. 5c) and no labeled cells are seen in the myocardium of the sinus venosus as demarcated by cardiac troponin I mRNA expression (data not shown). The septum primum does not express the transgene. At E12.5, the myocardium of the arterial pole of the heart (outflow tract), which overlies the endocardial cushions, does not express the transgene (Fig. 5d).

As the right and left ventricular and atrial chambers become established, the pattern of transgene expression is maintained in both right and left components of the embryonic ventricular and atrial segments and also in the atrio-ventricular canal (Fig. 5e). The outflow tract and inflow tract regions do not express the transgene; this pattern is maintained until the outflow tract starts to separate into right and left components (E13 onward).

**3F-*nlacZ*-9 transgene expression as a marker to follow the process of outflow tract septation (E13–E16).** As the outflow tract undergoes separation into aortic and pulmonary trunks (E13–15), the distal portion of the right ventricular segment shows no expression of *nlacZ* (E15; Fig. 5f). A sharp boundary between the  $\beta$ -galactosidase-negative cells and labeled myocardiocytes of the ventricular compartments is observed. This boundary coincides, ventrally, with the bulboventricular groove (Fig. 5f). As the outflow tract starts to “disappear” as a clearly identifiable segment of the heart, the endocardial cushions become fused and separated into right and left portions by the formation of the aorticopulmonary and conal septa. The myocardium surrounding

the semilunar valve swellings (Fig. 6a) and the conal ridges (Fig. 6b) is negative for the transgene as is the adjacent trabeculated portion of the embryonic right ventricle (Figs. 6c and 6d). The latter area corresponds to the embryonic right infundibulum. The negative area within the right ventricle increases in size with development (E13–E16). At the end of the process of arterial pole septation (E16) an area surrounding the left semilunar valve is negative for transgene expression (Fig. 6e), as is the outlet region of the right semilunar valve (Fig. 6f). These  $\beta$ -galactosidase-negative myocardial cells are, however, positive for cardiac troponin I mRNA (Figs. 6g and 6h).

**3F-*nlacZ*-9 transgene expression in the adult heart.** The hearts of 3F-*nlacZ*-9 mice show expression in both right and left atria and ventricles. Within the ventricular compartments, the right ventricle shows a patch of non-*nlacZ*-expressing cells in the distal part of the free wall (Fig. 7a). The outlet septum is also negative (Fig. 7c). In contrast, the left ventricular free wall and the interventricular septum are entirely positive. Only a thin rim in the subaortic portion at the level of the semilunar valve is negative (Fig. 7b). At the venous pole of the heart, myocardial structures such as the caval veins, pulmonary veins, coronary sinus, interatrial septum, and dorsal wall of the atria do not express the transgene (data not shown).

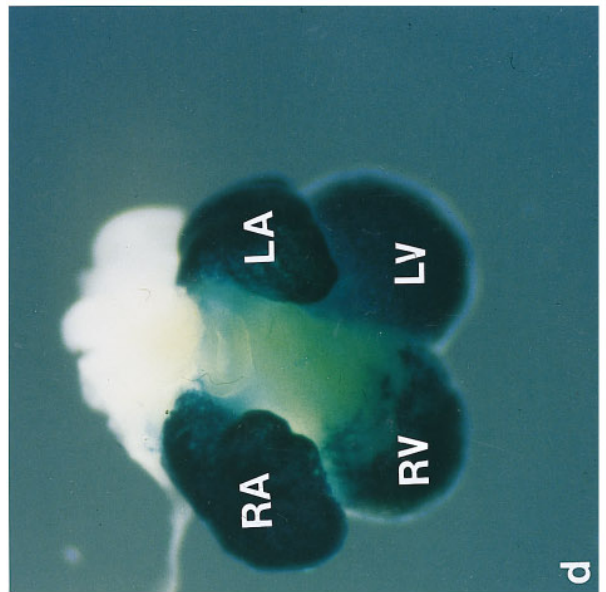
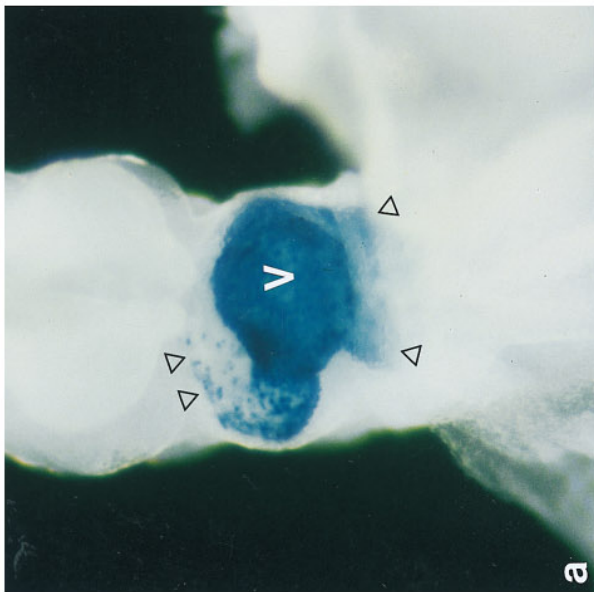
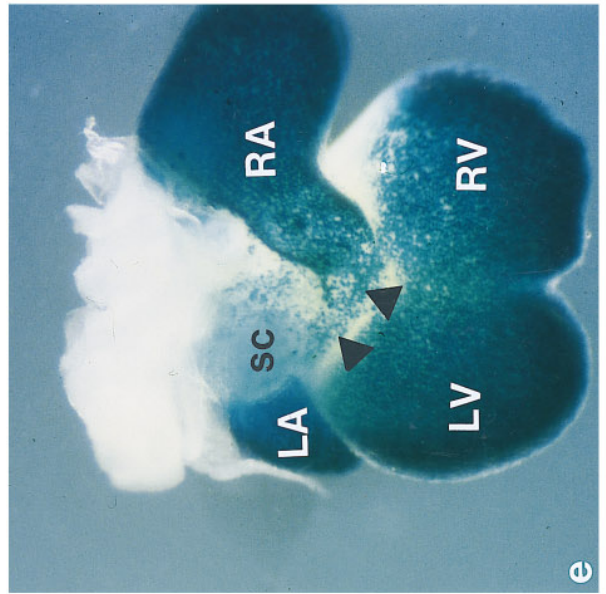
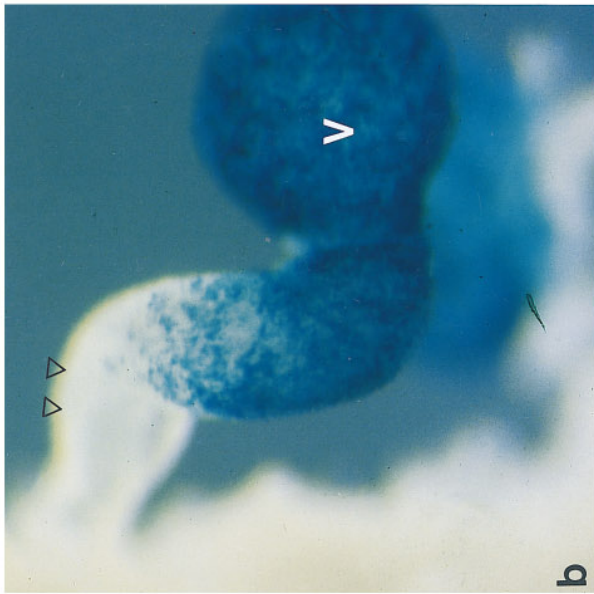
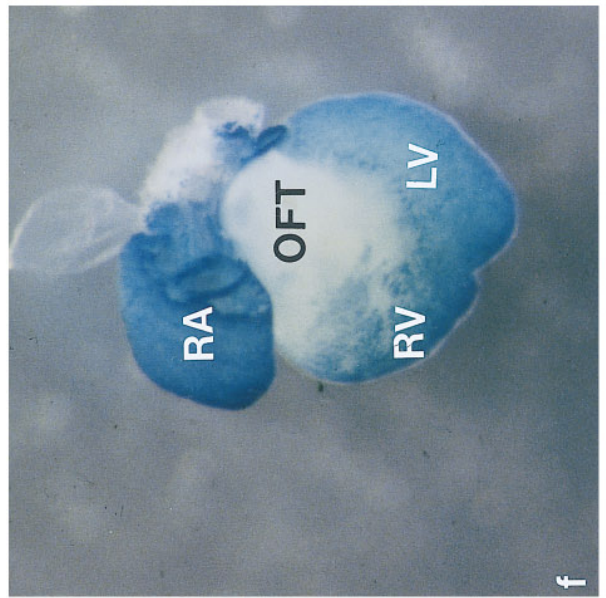
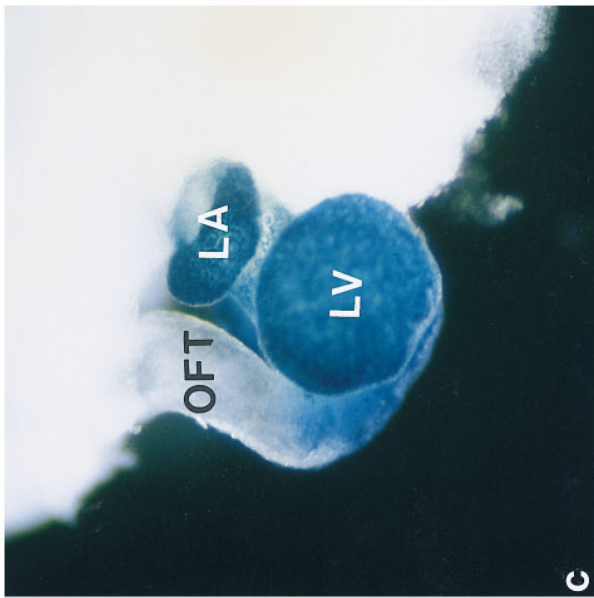
## DISCUSSION

### *Morphogenesis of the Cardiac Compartments: Transcriptional Regulation in the Early Heart*

In this study we show that two *nlacZ* transgenes with different regulatory elements from the myosin MLC1F/3F gene have distinct expression patterns in the mouse heart. One (3F-*nlacZ*-2E) shows striking left/right differences, with the right ventricle, left atrium, outflow tract, and inflow tract negative for transgene expression, while the second (3F-*nlacZ*-9) is expressed more extensively in the atria and ventricles, but the outflow and inflow tracts are negative. A developmental analysis shows that the early heart tube does not show regionalization of transgene expression;  $\beta$ -galactosidase-positive cells are similarly distributed throughout the linear heart tube. It is only after the onset of looping (E9) that regional differences begin to be apparent. In the chicken (Yutzey *et al.*, 1994; Yutzey and Bader, 1995) there is evidence from atrial myosin heavy chain gene ex-

**FIG. 5.** Developmental profile of transgene expression in 3F-*nlacZ*-9 mice. *In toto* localization of  $\beta$ -galactosidase in 3F-*nlacZ*-9 embryonic hearts. (a) At E8.5 the early looping heart shows a profile of transgene expression which is indistinguishable from that of the 3F-*nlacZ*-2E mice at the same stage (see Fig. 2c).  $\beta$ -Galactosidase expression is weaker at both poles of the cardiac tube (arrowheads) (b). At E9, transgene expression is negative in the arterial pole of the heart (arrowheads); and expression is still comparable to that of the 3F-*nlacZ*-2E transgene. At later stages of development (E10.5 (c); E12.5 (d)), the outflow tract (OFT) is negative for transgene expression, whereas both ventricles (LV, RV) and both atria (LA, RA) are positive. (e) A dorsal view at E12.5 shows that the inflow tract including the superior caval veins (sc) is negative (translucent blue overlying stronger expressing atrial region). Note that the atrioventricular canal (arrowheads) is also positive. (f) At E15, the right ventricular infundibulum (OFT) is  $\beta$ -galactosidase negative.





pression that a subpopulation of cells which will contribute to the atrial (posterior at this stage) compartment is already distinguishable in the linear heart tube as it forms. In the mouse, the myosin MLC2V gene has been reported to show early expression in future ventricular cells (O'Brien *et al.*, 1993). In the mammalian heart gradients of gene expression have been detected in the primitive cardiac tube, but it is only after looping of the heart that clearly defined compartment-specific gene expression is detected (Moorman and Lamers, 1994). The areas which appear as negative in our transgenic lines later in development may not be represented by an extensive cell population in the primitive cardiac tube. This is probable in the case of the outflow and inflow tracts and may extend to the adjacent right ventricle and left atrium, respectively. Regionalization of transcriptional potential, at this level of resolution, on the anterior/posterior axis of the primitive tube is not yet established.

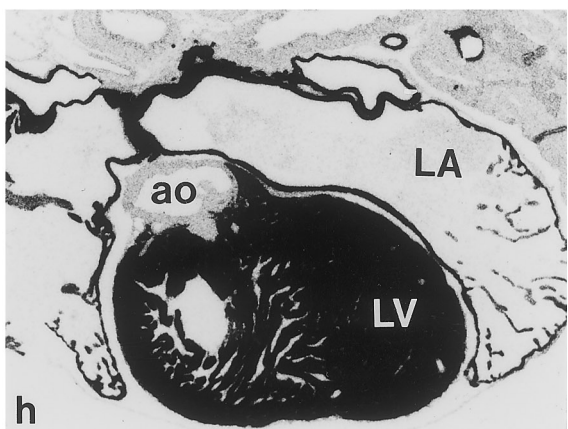
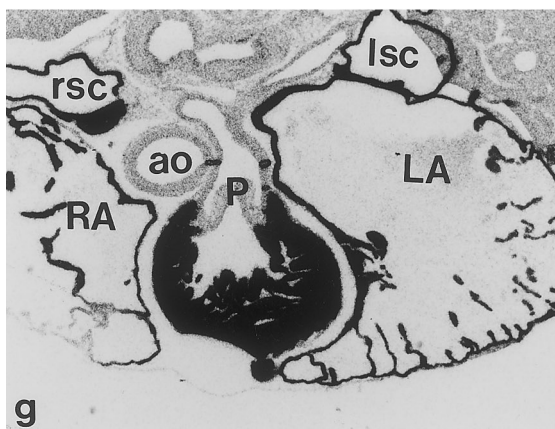
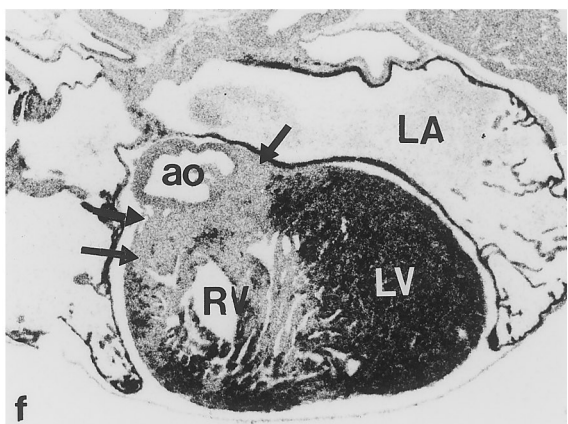
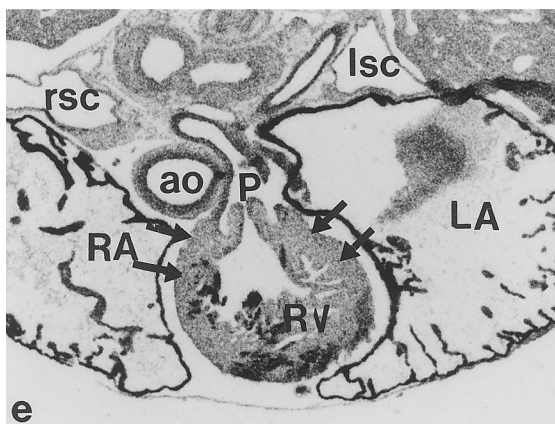
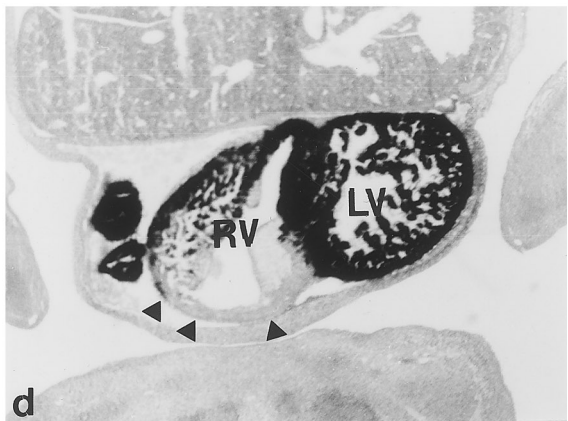
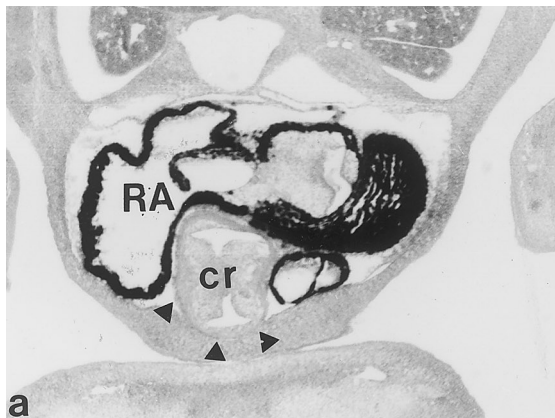
The results reported here indicate that, once looping is initiated, different regions of the heart, including left/right compartments, have different transcriptional potential.  $\beta$ -Galactosidase expression correlates with the level of *nlacZ* transcripts (Kelly *et al.*, 1995; this paper), confirming that these differences are at the transcriptional level. We conclude that regulatory elements in the transgenes, probably in the extended MLC3F promoter region (Kelly *et al.*, manuscript submitted; McGrew *et al.*, 1996), and the intronic enhancer are activated in different regions of the heart. Presumably, elements responsible for regional activation or repression of the transgenes are masked in the context of the endogenous gene. We are investigating the significance of these elements with respect to the expression pattern of the endogenous gene. That the regulatory elements are contained in the transgenes themselves, rather than coming from surrounding DNA at the site of insertion of the transgene, is suggested by the fact that different transgenic lines containing the same construct show similar expression patterns.

Recently, transgenic lines carrying regulatory sequences of a number of different myocardial genes have also been reported to show left/right differences in expression in the heart. Regulatory sequences from the cardiac  $\alpha$ -actin gene (Biben *et al.*, 1996) show a similar pattern of *nlacZ* transgene expression to that seen in the 3F-*nlacZ*-2E line. In contrast *lacZ* transgenes carrying regulatory sequences from the SM22 $\alpha$  (Li *et al.*, 1996), desmin (Kuisk *et al.*, 1996), and myosin MLC2V (Ross *et al.*, 1996) genes show expression

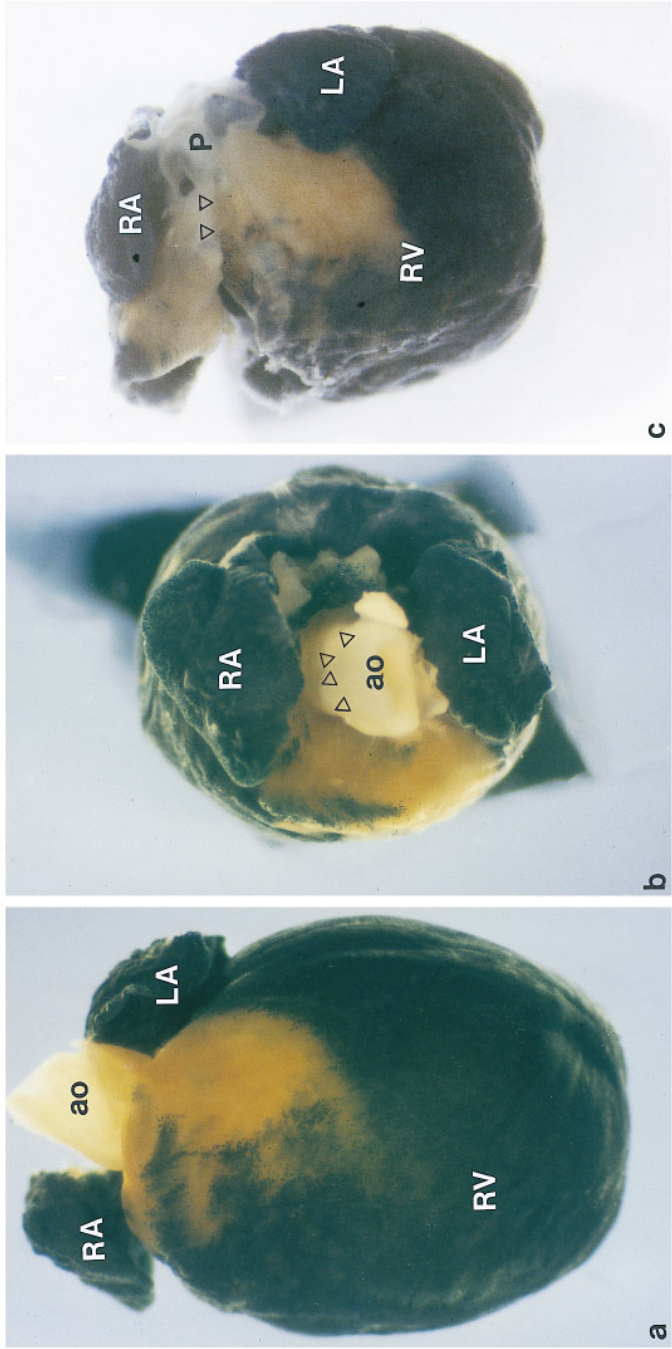
in the right ventricle and outflow tract rather than the left ventricle. Again, these differences in expression are clearly detected in the embryonic heart, once looping has occurred, although there is a suggestion that the MLC2V transgene may show earlier regionalization (Ross *et al.*, 1996). If this is the case, it would suggest that the right ventricular compartment can already be distinguished in the early heart tube. Precise comparison of developmental timing between lines showing right ventricular transgene expression and those described in this study, however, is necessary to clarify this point.

Not only transgenes, but also a few endogenous cardiac genes show some left/right differences in transcription. This has been reported for the MCK gene at the time of onset of expression in the mouse heart, which is relatively late (E14) (Lyons, 1994). MCK transcripts are first detectable in the right ventricle before extending to the whole myocardium. Conversely, the ANF gene, which is initially expressed throughout the myocardium, is down-regulated in the ventricles from about E14, where it remains detectable at very low levels in the left ventricle (Nemer *et al.*, 1986; Zeller *et al.*, 1987). Recently two sequences, e-HAND and d-HAND, which carry the basic-HLH transcription factor motif have been shown to be present in the early vertebrate heart (Srivastava *et al.*, 1995). In birds, antisense RNA experiments have shown that interference with both e-HAND and d-HAND arrests cardiac development at the looping heart tube stage (Srivastava *et al.*, 1995). Interestingly, in the mouse heart the e-HAND gene is not expressed in the right ventricle (Lyons, 1996; C. Biben and R. P. Harvey, unpublished data); again this restriction is detected as the heart begins to loop. A mutation in Nkx2.5, the vertebrate homologue of the *tinman* gene which is essential for heart (dorsal vessel) formation in *Drosophila* (Bodmer, 1993), interferes with the looping of the mouse heart and subsequent morphogenesis (Lyons *et al.*, 1995). In this mutant, e-HAND expression is abolished, suggesting that it may play a role in the looping phenomenon and related right-left regionalization (see Srivastava and Olson, 1996). Our results suggest that additional transcriptional factors which show regionalized expression are likely to exist, and their isolation and the subsequent study of their activation in the early heart should provide more information about the nature and origin of regional differences. Transgenes such as those reported here provide support for the existence of regional transcriptional domains and provide the first markers for

**FIG. 6.** Septation of the outflow tract in 3F-*nlacZ*-9 mice. Detection of  $\beta$ -galactosidase activity in serial transverse sections of an E14.5 heart (a–d). (a) At the distal level of the outflow tract the myocardium surrounding the endocardial cushions is negative for the transgene expression (arrowheads). (b) More proximally, where the cushions are not yet fused, the myocardium (arrowheads) surrounding the conal ridges (cr) is still negative, whereas the left ventricle (LV), atrioventricular canal (AVC), and atrial myocardium are positive. At the level of the boundary of the cushions, myocardium within the RV does not express  $\beta$ -galactosidase (c), and even more caudally, trabeculae show no transgene expression (arrowheads) (d). *In situ* hybridization used radiolabeled *lacZ* cRNA (e, f) and cardiac troponin I cRNA (g, h) probes in serial sections of an E16 heart. *lacZ* is not expressed in the myocardium surrounding the right semilunar valve or the left semilunar valve (arrows), respectively (e, f). Furthermore, the right superior cava vein (rsc) and the left superior cava vein (lsc) are negative for transgene expression (e, f) but positive for cardiac troponin I mRNA (g, h). RV, right ventricle; RA, right atrium; LA, left atrium; ao, aorta; P, pulmonary trunk.







**FIG. 7.** Transgene expression in the adult 3F-nlacZ-9 mice. In toto localization of transgene expression in adult 3F-nlacZ-9 mice. (a) A ventral view of the heart shows that the right ventricular infundibulum is negative for the transgene. (b) In a cephalic view myocardium which is negative for the transgene surrounds the aortic valvular orifice (arrowheads). (c) Dissection of the adult heart throughout the pulmonary valve shows that the outlet septum, i.e., the septum separating the aortic and the pulmonary outlets (arrowheads), is also negative for the transgene. RV, right ventricle; LV, left ventricle; RA, right atrium; LA, left atrium; P, pulmonary trunk; ao, aorta.



left versus right differences as well as further myocardial subdivisions such as regionalization within the right ventricle. Although it is difficult to draw conclusions about possible lineage differences in the cell populations which make up these compartments from observations based on gene expression, since the transcriptional status of the transgene may fluctuate, the results reported here tend to suggest that early heart looping and transcriptional compartmentalization are linked processes.

### **Insights into Specific Aspects of Cardiac Morphogenesis Provided by the Patterns of MLC3F Transgene Expression**

**Septation of the atrioventricular junction.** It is well established that the working atrial myocardium is continuous with the myocardium of the ventricles through the atrioventricular junction during early cardiac development (Anderson *et al.*, 1978; Wessels *et al.*, 1991). At this stage, the atrioventricular junction myocardium shows a slow impulse propagation, thus substituting for valve function and overcoming the necessity for insulation of the atrial and ventricular myocardium with fibrous tissue (Moorman and Lamers, 1994; De Jong *et al.*, 1992, 1993). The insulation of the atrial and ventricular myocardial masses occurs concomitantly with the development of the atrioventricular valves (Wessels *et al.*, 1996).

In this study we show that the atrioventricular canal is positive for the expression of the transgene in the 3F-*nlacZ*-2E line while the left atrium is negative and the right atrium is positive. With development,  $\beta$ -galactosidase-positive cells are also observed in the lower rim of the left atrial myocardium. These findings show for the first time that a subpopulation of the atrial myocardium, characterized by low expression of connexin 43 (Van Kempen *et al.*, 1996) and thus a slow impulse conduction (De Jong *et al.*, 1987), has a distinct transcriptional regulation. These data support the notion that the former atrioventricular myocardium becomes incorporated into the lower rim of the atrial walls (Wessels *et al.*, 1996), maintaining a distinct pattern of gene expression.

Lamers *et al.* (1995) have suggested that, in man, the tricuspid valve is derived entirely from the right ventricular myocardium. Although these authors were capable of distinguishing atrial and ventricular myocardial cell subpopulations by their distinct expression of either  $\alpha$ - or  $\beta$ -MHC isozymes, they could not distinguish between right and left ventricular components. In this study we show that  $\beta$ -galactosidase-positive cells are observed in the lesser curvature of the heart; as development proceeds, these cells underlie the fibrous tissue of the right atrioventricular anterior leaflet. These myocardial cells show expression of the 3F-*nlacZ*-2E transgene and  $\beta$ -MHC mRNA (ventricular) but not  $\alpha$ -MHC mRNA (atrial). This suggests that a small population of cells from the lesser curvature contributes to the muscular component of the tricuspid valve leaflets. It remains to be established whether these observations in mice can be made in man, since there are differences in the adult

atrioventricular valve morphology between these species (Icardo *et al.*, 1993).

### **Contribution of the Embryonic Outflow Tract to the Adult Ventricular Chambers**

An important and unsolved issue in cardiac embryology is whether the embryonic outflow tract disappears or is partially reabsorbed into the ventricular compartment (Goor *et al.*, 1972; Pexieder, 1995). Similarly, controversy still exists about the developmental process leading to the formation of an independent arterial connection from the left ventricle (Pexieder, 1995). These questions remained obscure because of the lack of markers distinguishing right and left ventricular compartments from the embryonic outflow tract. We present here the first detailed description of differential gene (transgene) expression between the outflow tract and the embryonic right ventricle that allows us to approach the questions of outflow tract fate and septation. Our data suggest that the embryonic outflow tract contributes mostly to the right ventricular infundibulum, while a minor portion remains surrounding the left semilunar valve. The outlet septum of the right ventricle also lacks expression of the transgene, suggesting that this structure is derived from the embryonic outflow tract. A complementary image (positive outflow tract is provided by another transgenic MLC1V line that we have developed in the laboratory (R. Kelly, unpublished data). Our data are in agreement with observations showing that muscularization of the endocardial cushions by ingrowth of outflow tract myocardial cells contributes to the formation of the outlet septum (Lamers *et al.*, 1995; Franco *et al.*, 1996).

Another intriguing aspect of heart formation is the origin and development of the atrial chambers which occurs relatively late during cardiogenesis. In the 3F-*nlacZ*-9 line the inflow tract is also  $\beta$ -galactosidase negative. Detailed analysis of transgene expression in the developing atrial myocardium in relation to the extension of the pericardial cavity should further our understanding of this aspect of inflow tract ontogeny. Diverse aspects of cardiac morphogenesis can therefore be reassessed using the transcriptional subdomains revealed by these and other transgenic lines.

## **ACKNOWLEDGMENTS**

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